

DNA for Evaluating the Progression Potential of Cervical Lesions

**RELATED APPLICATION**

This application is a continuation of Serial No. 09/308,984, filed September 3, 1999, now

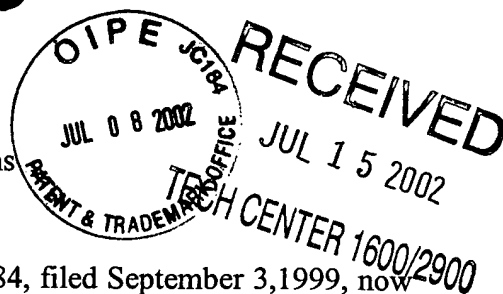
U.S. Patent No.

This invention relates to [DNA] nucleic acid molecules suitable for evaluating the progression potential of cervical lesions, and to polypeptides encoded [coded] by such a [DNA] nucleic acid molecules. Furthermore, this invention concerns antibodies directed against the polypeptides. Moreover, it covers the use of the [DNA] nucleic acid molecules and the polypeptides as well as a kit suitable for evaluating the progression of cervical lesions.

[The] I[i]nvasive cervical carcinoma usually follows from a precancerosis. Precancerosis cover a wide range of lesions referred to as mild to severe dysplasias (CIN1 to CIN3) pertaining to histopathology. CIN1 lesions frequently regress spontaneously and usually need not be treated. On the other hand, these lesions can persist over years or change into a more severe lesion, e.g. CIN3, or into a [higher] more invasive carcinoma. A cytological method has been used for the diagnosis of cervical smears for about 50 years, by means of which dysplastic cells can be detected in cervical smears. This method is generally known as the "Pap test". The "Pap test" contributed to the fact that the incidence of the cervical carcinoma could be reduced significantly in the past.

Several years ago, it was also found that the presence of dysplastic lesions and cervical carcinomas correlates with the detection of cervical carcinoma-associated human papilloma viruses, e.g. HPV 16 or HPV 18. The detection of antibodies against the viral HPV oncoproteins E6 and E7 in patient serum by means of ELISA or comparable methods is also possible as a detection of premalignant or malignant cervical diseases. It is also known [discussed] that certain chromosomal deletions are associated with an increased risk of malignant transformation of the corresponding precancerosis. Morphological changes of cells and cell nuclei also correlate with malignant progression. These changes can be detected by means of cytometry.

Nevertheless, it is not possible by either the "Pap test" or the detection of oncogenic HPV types to make a prognosis regarding the further development of individual lesions. This also applies to the detection of HPV-specific antibodies in patient serum. By means of this serological method it is rather only possible to detect patients who already suffer from a carcinoma. Therefore, the antibody detection method cannot be considered a supplement to the



present precaution but only to the manifestation of the finding that a carcinoma had developed already. In addition, the genetical analysis and cytometric approaches include the drawback that they are technically very complicated and therefore are of [no significance] not useful for routine diagnostics.

Therefore, it is the object of the present invention to provide a product by which the progressive [progression] potential of cervical lesions can be evaluated reliably.

This is the subject of the invention set forth in the disclosure which follows. [According to the invention this is achieved by the subject matters defined in the claims.]

Thus, the subject matter of the present invention relates to [a] an isolated nucleic acid molecule suitable for evaluating the progressive [progression] potential of cervical lesions. Such a nucleic acid molecule can be obtained [provided] by common methods. A method is preferred [favorable] in which RNA from early and late passages of HPV-immortalized cells is isolated and the RNAs characteristic of [for] of the early passages and late passages, respectively, and expressed in markedly differing amounts, respectively, are identified and provided as DNA or RNA.

The present invention is based on the applicant's finding that late passages of HPV-immortalized cells cause tumors in naked mice, whereas early passages of such cells are not capable of doing [to do] so. The inventor also discovered that in late passages of HPV-immortalized cells certain RNAs are [can be] detected more strongly than is the case in early passages of such cells.

For the provision of a nucleic acid according to the invention RNA from early and late passages of HPV-immortalized cells are [can be] isolated. Early passages are e.g. passage 20-60, and late passages start from e.g. 130. For example, HPV 16-immortalized, human preputial keratinocytes, HPK-IA cells can be used as cells (cf. Dürst, M. et al., Oncogene 1/3 (1987), 251-256). The RNA of the early and late passages can be compared with each other and differences can be determined which are characteristic of the early passages and late passages, respectively. To this end, it is favorable to subject the RNA to [a]reverse transcription . In this case, it is advantageous to use what are known as [is called] anchor [anchorage] primers, i.e. oligo-d(T) primers which following a sequence of 11-15 thymidine bases have two more bases at the 3' end and thus recognize in well-known [calculated] fashion the transition from the 3' end of an mRNA to the poly-A tail where they bind. The resulting cDNA can be subjected to [an]

amplification in a PCR method. For this purpose, it is favorable to use common "arbitrary" primers together with the above anchor [anchorage] primers. The amplified cDNA can then be subjected to [a] denatured polyacrylamide gel electrophoresis. In this step, cDNA bands are identified which have a differing intensity in the cDNA samples to be compared with one another, i.e. RNA isolates from the early and late passages of HPV-immortalized cells. These cDNA bands can be isolated from the gel and subjected to another round of [above] amplification. Moreover, they can be cloned and their sequences can be determined. A person skilled in the art is familiar with the above methods. By way of supplement reference is made to the following literature (cf. Liang et al., Cancer Research 5, (1992), 6966-6968; Liang et al., Science 257, (1992), 967-971; Liang et al., Nucleic Acids Research 21, (1993), 3269-3275).

Both cDNA and RNA are examples of nucleic acid molecules in accordance with the invention, with cDNA being preferred. [An above [c]DNA is a nucleic acid according to the invention. The latter is also is a corresponding RNA, a [c]DNA being preferred.] A (c)DNA which comprises a base sequence of fig. 1 of fig. 2 or a sequence differing therefrom by one or several base pairs is particularly preferred. The (c)DNA of fig. 1 was deposited as C4.8 with the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) {Germany-type collection of microorganisms and cell cultures} under DSM 11197 on October 4, 1996. Furthermore, the (c)DNA of fig. 2 was deposited as C21.7 with the DSMZ under DSM 11198 on October 4, 1996. A nucleic acid molecule according to the invention is described as DNA by way of example below.

A DNA according to the invention can be present in a vector, [and] an expression vector, respectively. The person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli these [are] include e.g. pGEMEX, pUC derivatives, pGEX-2T, pQE-8 and pet3d. For the expression in yeast e.g. pY100 and Ycpad1 have to be mentioned, while e.g. pKCR, pEFBOS, cDM8 and pCEV4 have to be indicated for the expression in animal cells. The baculovirus expression vector pAcSGHisNT-A is particularly suitable for the expression in insect cells.

The person skilled in the art knows suitable cells [to] which express [a] DNA according to the invention, which is present in an expression vector. Examples of such cells comprise the E. coli strains HB101, DH, x1776, JM101, JM109, SG13009 and BL21, the yeast strain

Saccharomyces cerevisiae and the animal cells L, 3T3, FM3A, CHO, COS, Vero and HeLa as well as the insect cells sf9.

The person skilled in the art knows in which way a DNA according to the invention has to be inserted in an expression vector. He is also familiar with the fact that this DNA can be inserted in combination with a DNA coding for another polypeptide, so that the DNA according to the invention can be expressed in the form of a fusion protein.

In addition, the person skilled in the art knows conditions of culturing transformed cells and transfected cells, respectively. He is also familiar with methods of isolating and purifying the polypeptide expressed by the DNA according to the invention. Thus, such a polypeptide, which may also be a fusion polypeptide, also represents a feature [subject matter] of the present invention. A [An above] polypeptide in accordance with the invention [preferably] comprises the amino acid sequence of fig. 1 or a sequence differing therefrom by one or several amino acids.

A further aspect [subject matter] of the present invention relates to an antibody directed against an above polypeptide and fusion polypeptide, respectively. Such an antibody can be prepared by common methods. It may be polyclonal and monoclonal, respectively. For its preparation it is favorable to immunize animals – particularly rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody – with an above (fusion) polypeptide. Further “boosters” of the animals can be effected with the same (fusion) polypeptide. The polyclonal antibody can then be obtained from the animal serum and egg yolk, respectively. For the preparation of the monoclonal antibody, animal spleen cells are fused with myeloma cells.

The present invention enables the reliable evaluation of the progressive [progression] potential of cervical lesions. By means of an antibody according to the invention it can be determined whether cervical smears contain polypeptides which are characteristic of early or late passages of HPV-immortalized cells. Furthermore, it is possible to detect an autoantibody directed against the polypeptide present in the body by means of a polypeptide according to the invention. Both detections can be made by common methods, particularly a Western blot, an ELISA, an immunoprecipitation or by immunofluorescence. In addition, it is possible by means of a nucleic acid molecule according to the invention, particularly [a] DNA and primers derived therefrom, to detect whether RNA which is characteristic of early or late passages of HPV-immortalized cells is present in cervical smears. This detection can be made [as usual] in

accordance with known methods, particularly in a Southern blot. By means of the present invention it is thus possible, to make an early diagnosis [statement] of whether a cervical carcinoma is forming.

Furthermore, the present invention is useful for preventing [suitable to take steps against] the formation of a cervical carcinoma. By means of an antibody according to the invention it is possible to inhibit a polypeptide which is characteristic of late passages of HPV-immortalized cells. Moreover, a nucleic acid molecule according to the invention, particularly [a] DNA, can be used to inhibit such a polypeptide. To this end, the nucleic acid molecule is used for the [expression] inhibition of expression of the gene coding for the polypeptide, e.g. as a basis of preparing anti-sense oligonucleotides.

To carry out the invention, particularly as regards the diagnostic aspect, a kit is also provided. It contains one or several nucleic acid[s] molecules, polypeptides and/ or antibodies according to the invention. In particular, it comprises those nucleic acid[s] molecules and/or polypeptides which are said to be preferred above. In addition, the kit contains conventional excipients such as carriers, buffers, solvents and controls. The kit is also the subject matter of the present invention.

The present invention is explained by the examples which follow [below examples].

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

- Fig. 1            shows the base sequence of (c)DNA C4.8 according to the invention. In addition, the amino acid sequence of the polypeptide encoded by (c)DNA C4.8 is indicated,
- Fig. 2            shows the base sequence of (c)DNA C21.7 according to the invention.
- Example 1:      Preparation of cDNAs C4.8 and C21.7 according to the invention.

Whole RNA was isolated in each case by the known guanidine thiocyanate (GTC) method from early passages, i.e. passage 49, and late passages, i.e. passage 359 and p389, of the HPV-immortalized cell line HPK-IA [see above]. The whole RNA was subjected to a conventional DNase reaction, using commercially available RNase-free DNase purchased from PROMEGA [the RQ1-RNase free DNA of PROMEGA having been used].

The resulting DNase-free whole RNA was subjected to [a] reverse transcription, [method what is called anchoring primers having been used as primers] using anchor primers, as are described supra. Following a sequence of 11-15 thymidine bases, these primers have two more

bases at the 3' end, e.g. AA, AC, AG, CA, CC, CG, GA, GC, GG, AT, CT, GT, so that the primers are bound directly at the transition from the mRNA to the poly-A tail.

The conditions for reverse transcription were as follows:

RNAsine 20 u/μl	1.0 μl
dNTP-Mix (2.5 mM)	1.2 μl
MMLV reverse transcriptase 300 u/μl	2.5 μl
0.1 M DTT	5.0 μl
whole RNA 250 ng/μl	5.0 μl
T <sub>12</sub> VV primers (V=A, C or G), 25 μM	5.0 μl
5 x RT buffer*	10.0 μl
dH <sub>2</sub> O	20.3 μl

50.0 μl

\* : 5 x RT buffer: 250 mM Tris-HCl (pH 7.6)  
375 mM KCl  
15 mM MgCl<sub>2</sub>

The whole RNA was denatured prior to the reaction at 70-80°C for 5 minutes, then quenched on ice and fed to the reaction vessel. All of the other components were mixed at 0°C and added to the whole RNA. [ , u] Ultimately all was coated with mineral oil and incubated in a water bath at 37°C for 45-60 minutes. Finally, the reaction was stopped by inactivating the enzyme at 95°C (5 minutes). Having terminated the reaction, the RT batches were frozen at --20°C up to their use.

The resulting cDNA was subjected to a PCR method. To this end, 20 μl batches were made, each having 2 μl of the above reverse transcription batch as template (1/10 of the reaction volume, corresponding to the equivalent of 25-50 ng cDNA/PCR batch). The other components (cf. below) were prepared as "master mix" and then added. A PCR reaction batch was composed as follows:

RT batch (prepared)	2.0 μl
Mix:	
10 x PCR buffer*	2.0 μl
10-mer arbitrary primer 5 μM	2.5 μl

T <sub>12</sub> VV primers (V=A, C or G), 25 µM	2.0 µl
dNTP mix (2.5 mM in toto)	0.4 µl
50 mM MgCl <sub>2</sub>	0.7 µl
Taq DNA polymerase 20 U/µl	0.2 µl
α – <sup>32</sup> P-dCTP	0.1 µl
dH <sub>2</sub> O	10.6 µl
	20.0 µl

\*: 10x PCR buffer: 200 mM Tris-HCl (pH 8.55)  
 160 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
 magnesium: optimum concentration  
 is adjusted with 50 mM MgCl<sub>2</sub>

The 10-mer arbitrary primer is e.g. “AGC CAG CGA A” (AP-1) or “GCA ATC GAT G” (AP-6). The reaction was carried out in a DNA thermocycler (Perkin-Elmer Gen-Amp 9600) with the following program steps:

Program 1:	denaturation	95°C, 3 minutes	1 cycle
Program 2:	denaturation	95°C, 15 seconds	a maximum
	primer annealing	40°C, 2 minutes	of 30
	primer extension	72°C, 30 seconds	cycles
Program 3:	Primer extension	72°C, 5 minutes	1 cycle

Having terminated the PCR method, the batches were applied to a denaturing 4.5-6% polyacrylamide gel. By comparing [comparison] the cDNA bands from the early and late passages of the HPK-IA cells, those could be identified which were different, i.e. were represented in the late passages much more than in the early ones.

These cDNA bands were used for another amplification. To this end, they were cut out of the polyacrylamide gel and used in another PCR method. The PCR batch was composed as follows:

10 x PCR buffer	5.0 µl
10-mer arbitrary primer 5 µM	5.0 µl
T <sub>12</sub> VV primer (V=A, C or G), 25 µM	5.0 µl
dNTP mix (25 mM in toto)	1.2 µl

50 mM MgCl <sub>2</sub>	1.5 µl
Taq-DNA polymerase 20 U/µl	1.0 µl
dH <sub>2</sub> O	31.5 µl
	50.0 µl

The PCR reaction was carried out under the same conditions and with the same program sequence as the first PCR reaction.

Having terminated the PCR reaction, the batches were separated on a 1% agarose gel, and the desired DNA bands were cut out of the gel. Thereafter, the DNA bands eluted from the agarose pieces by using what is called "GenElute" columns (SUPELCO company).

The resulting cDNA was cloned into the cloning vector pCRII using the "TA cloning kit" (INVITROGEN company). Resulting clones were sequenced [determined] by means of the "T7 sequencing kit" (PHARMACIA company) [as regards their sequence]. The cDNAs C4.8 and C21.7 according to the invention were obtained.

Example 2: Comparative studies using cDNAs C4.8 and C21.7 according to the invention.

- a) The whole RNA isolated according to Example 1 from early and late passages of HPK-IA cells was subjected to a denaturing 4.5-6% polyacrylamide gel electrophoresis. Thereafter, a conventional Northern blot was carried out, the RNA being transferred to "Gene Screen-Plus" nylon membranes. <sup>32</sup>P-labeled cDNAs C4.8 and C21.7 according to the invention were used for hybridization.

[It showed] The results indicated that the cDNA according to the invention react much more strongly with the RNA from the late passages of the HPK-IA cells than was the case with the early passages.

- b) Furthermore, RNA-RNA *in situ* hybridization's were made with freezing sections from cervical tissues, namely normal epithelial tissue, premalignant lesion and carcinoma to evaluate the condition of the tissue. RNA probes were used as hybridization probes, which were obtained from the cDNAs C4.8 and C21.7 according to the invention. To this end, the latter were linearized and RNA was synthesized by adding the corresponding RNA polymerase, preferably SP6 or T7, and <sup>32</sup>P-rUTP. The RNA-RNA *in situ* hybridization with the above tissue was carried out under stringent conditions, e.g. at 60°C.



It turned out that [a] strong hybridization was only obtained in connection with the cervical carcinoma.

However, the hybridization was very low in the case of normal epithelial tissue.

The above data underline that the present invention is perfectly suited to detect potentially malignant cells in a cervical smear.

## CLAIMS

1. A nucleic acid suitable for evaluating the progression potential of cervical lesions, wherein the nucleic acid is obtainable by a process in which RNA from early and late passages of HPV-immortalized cells is isolated and the RNAs characteristic for the early passages and late passages, respectively are identified and provided as DNA or RNA.
2. The nucleic acid according to claim 1, characterized in that the nucleic acid is provided as DNA.
3. The nucleic acid according to claim 1, characterized in that the nucleic acid comprises the base sequence of fig. 1 or a sequence differing therefrom by one or several base pairs.
4. The nucleic acid according to claim 1, characterized in that the nucleic acid comprises the base sequence of fig. 2 or a sequence differing therefrom by one or several base pairs.
5. A polypeptide, comprising an amino acid sequence which is coded by the nucleic acid according to claim 1.
6. The polypeptide according to claim 5, characterized in that the polypeptide comprises the amino acid sequence of fig. 1 or a sequence differing therefrom by one or several amino acids.
7. A process for the preparation of the nucleic acid according to claim 1, in which RNA is isolated from early and late passages of HPV-immortalized cells and the RNA characteristic for the early passages and late passages, respectively, are identified and provided as DNA or RNA.
8. An antibody directed against the polypeptide according to claim 4.
9. Use of the nucleic acid according to claim 1 as a reagent for diagnosis and/or treatment.
10. Use of the polypeptide according to claim 5 or 6 as a reagent for diagnosis.
11. Use of the antibody according to claim 8 as a reagent for diagnosis and/or treatment.
12. Kit comprising at least one nucleic acid molecule to claim 1, and a conventional auxiliary agent.

### **ABSTRACT OF THE DISCLOSURE**

The present invention relates to a nucleic acid which is suitable for evaluating the progression potential of cervical lesions, wherein the nucleic acid is obtainable by a process in which RNA from early and late passages of HPV-immortalized cells is isolated and the RNAs characteristic for the early passages and late passages, respectively, are identified and provided as DNA or RNA. Furthermore, the present invention concerns polypeptides coded by such a nucleic acid. In addition, it covers antibodies directed against the polypeptides. Moreover, it relates to the use of the DNA and the polypeptides as well as a kit suitable for evaluating the progression of cervical lesions.